CVI
Journals.ASM.org

Development of Immunochromatography-Based Methods for Detection of Leptospiral Lipopolysaccharide Antigen in Urine

Dian Widiyanti,[a,e] Nobuo Koizumi,[b] Takashi Fukui,[c] Lisa T. Muslich,[a] Takaya Segawa,[a] Sharon Y. A. M. Villanueva,[a] Mitsumasa Saito,[a] Toshiyuki Masuzawa,[c] Nina G. Gloriani,[d] Shin-ichi Yoshida[a]

Department of Bacteriology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan[a]; Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan[b]; Laboratory of Microbiology and Immunology, School of Pharmaceutical Sciences, Chiba Institute of Sciences, Chiba, Japan[c]; Department of Medical Microbiology, College of Public Health, University of the Philippines—Manila, Manila, Philippines[d]; Department of Microbiology-Parasitology, Faculty of Medicine, YARSI University, Jakarta, Indonesia[e]

Leptospirosis is an infectious disease caused by the spirochete bacteria *Leptospira* spp. and is commonly found throughout the world. Diagnosis of leptospirosis performed by culture and microscopic agglutination tests is laborious and time-consuming. Therefore, we aimed to develop a novel immunochromatography (ICG)-based method for detecting *Leptospira* antigen in the urine of patients and animals. We used the 1H6 monoclonal antibody (MAb), which is specific to the lipopolysaccharide (LPS) that is common among *Leptospira* spp. The MAb was coupled to 40-nm-diameter colloidal gold, and the amounts of labeled antibody and immobilized antibody were 23 μg and 2 μg per test, respectively. Several strains of *Leptospira* and other bacterial species were used to evaluate the sensitivities and specificities of the assays we developed. The detection limit of the assays was 10^6 cells/ml when disrupted whole bacterial cells were used. The assays were *Leptospira* specific since they did not cross-react with non-*Leptospira* bacteria used in the study. Application of diagnostic assays was done on the urine samples of 46 *Leptospira*-infected hamsters, 44 patients with suspected leptospirosis, and 14 healthy individuals. Pretreatment of the urine samples by boiling and centrifugation (for ultrafiltration and concentration) eliminated nonspecific reactions that occurred in the assay. The sensitivity and specificity of the ICG-based lateral flow assay (LFA) were 89% and 87%, respectively, which were higher than those of the dipstick assay, which were 80% and 74%, respectively. In summary, this ICG-based LFA can be used as an alternative diagnostic assay for leptospirosis. Further development is still necessary to improve the assay.

The genus *Leptospira*, belonging to the order *Spirochaetales* and family *Leptospiraceae*, comprises spiral-shaped bacteria that are 0.1 μm in diameter and 6 to 20 μm in length and that have hooked ends (1). *Leptospira* organisms are Gram negative and obligately aerobic (2). Infection in humans or animals might happen by penetration of *Leptospira*, excreted by infected host animals into the environment, through a wound or mucous membrane. Signs and symptoms of leptospirosis in humans range from mild flu-like symptoms to jaundice (hepatic dysfunction), oliguria or anuria (renal failure), and hemoptysis (lung hemorrhage), which can lead to death (3).

Several assays can be applied for leptospirosis diagnosis. The World Health Organization (WHO) has specified standard requirements for leptospirosis patients, such as sufficient growth of leptospires from a normally sterile organ, a clear amplified DNA band in PCR, and a 4-fold increase of titers between acute- and convalescent-phase sera in a microscopic agglutination test (MAT) (4). Unfortunately, MAT is laborious and time-consuming, and PCR is expensive due to the need for sophisticated equipment. Because leptospirosis is commonly found in developing or underdeveloped countries, there is a need for rapid, reliable, and inexpensive diagnostic kits. Diagnostic assays were developed several years ago, such as flow-through (5), IgM dipstick (6), immunofluorescence (7), and latex agglutination (8), which detected the presence of antibodies in human serum samples. Nevertheless, the sensitivity and specificity of these methods were low when performed during the early stage of infection with *Leptospira* because the appropriate immune response might not yet have been elicited by the time of specimen collection. For example, the dipstick assay (6), which can detect the presence of IgM and is often used in the initial screening of leptospirosis, has low sensitivity when applied to patient serum samples (9). An antigen detection assay might offer an effective solution to this difficulty, because antigen can be detected earlier after infection (10). Assays for the detection of *Leptospira* antigen and DNA are still being developed (11, 12). An immunochromatography (ICG)-based assay might be a solution because it is inexpensive, rapid, and easy to perform. An ICG-based assay for the detection of bacterial antigen in clinical samples has been performed on several bacterial species, such as *Legionella pneumophila* (13), *Streptococcus pneumoniae* (14), and *Neisseria meningitidis* (15). In our study, we tried to develop an ICG-based assay for antigen detection in *Leptospira*, which could be applied in areas where leptospirosis infection is endemic and that is applicable for detecting antigen in urine samples.

MATERIALS AND METHODS

Bacteria and culture. The bacterial species used in this study are listed in Table 1. These bacteria were cultured in modified Korthof's medium (16) for *Leptospira* spp., brain heart infusion (BHI) broth (Difco) for *Streptococcus* spp. and *Enterococcus* spp., selective buffered charcoal yeast extract α (BCYEα) for *Legionella* spp., and Luria-Bertani (LB) medium for *Escherichia coli* and *Pseudomonas* spp. These organisms were then used to

Received 13 December 2012 Returned for modification 21 January 2013
Accepted 27 February 2013
Published ahead of print 6 March 2013
Address correspondence to Dian Widiyanti, dian@bact.med.kyushu-u.ac.jp.

doi:10.1128/CVI.00756-12

TABLE 1 List of organisms used in this study

Bacterial species	Strain	Serovar or serogroup
Leptospira interrogans	Akiyami A	Autumnalis
	Hond Utrecht IV	Canicola
	K64	Manilae
	K5	Grippotyphosa
	Ictero no. 1	Icterohaemorrhagiae
	K37	Losbanos
	Hebdomadis	Hebdomadis
Leptospira borgpetersenii	Poi	Poi
	Perepelitsin	Tarassovi
	K6	Javanica
Leptospira biflexa	Patoc 1	Patoc
Escherichia coli	K-12 MG 1655	
	C16	
	C17	
Legionella pneumophila	Philadelphia-1 (ATCC 33152)	
Enterococcus faecalis	Portland (ATCC 29212)	
Pseudomonas aeruginosa	PAGU 221	
Streptococcus pneumoniae	ATCC 1127	
Serratia marcescens	J1	
	J5	
Borrelia burgdorferi	B31	
Borrelia afzelii	P/Gau	

examine the specificity and sensitivity of the assays developed or as infection agents in hamsters (*Leptospira* spp. only).

Monoclonal antibody production. Six-week-old BALB/c mice were primed intraperitoneally with 0.2 ml of a mixture of equal volumes of 0.1 mg of the heat-killed *Leptospira interrogans* serovar Icterohaemorrhagiae strain RGA (1.0×10^8 cells/ml in phosphate-buffered saline [PBS]) and Freund's complete adjuvant. The mice were immunized two more times at 1-week intervals using the same immunogen and the same route, but instead with Freund's incomplete adjuvant. Three days after the last booster, the mice were sacrificed. Hybridomas were generated following the fusion of splenocytes with P3-X63-Ag8.653 myeloma cells, and selected cultures were grown following standard procedure (17). Hybridomas were screened for the secretion of the desired antibodies with an enzyme-linked immunosorbent assay (ELISA) and Western blotting using homologous sonicated antigen. Positive hybridoma cells were cloned using limiting dilution to obtain antibodies from a single cell. Hybridoma culture supernatants or ascitic fluid, both of which were harvested after *in vivo* culture of hybridoma, were used as the 1H6 monoclonal antibody (MAb) source. Purification of protein from hybridoma was carried out by ammonium sulfate precipitation, followed by affinity chromatography (18) through a HiTrap Protein G HP column (GE Healthcare) in the presence of 1.5 M glycine (pH 9.0). Purified antibody was analyzed by SDS-PAGE, and quantitative measurement was determined by UV absorption (18). The immunoglobulin subclass was determined using a mouse monoclonal antibody isotyping kit (GE Healthcare), following the manufacturer's instructions.

Antigen specificity of MAbs. Specificity of the generated MAb, 1H6, was tested by immunoblotting against bacterial antigens, including the lipopolysaccharide (LPS) of several bacterial species. The LPS was extracted using chloroform-methanol extraction (19), followed by silica column chromatography (18) using Iatrobeads 6RS-8060 (Iatron Laboratories, Inc.). LPS was analyzed by SDS-PAGE with Pro-Q Emerald staining (Invitrogen). Immunoblotting of LPS was performed using the 1H6 MAb.

Animal and human urine. Four-week-old golden Syrian hamsters (Japan SLC, Inc., Hamamatsu, Japan) were infected with *L. interrogans* serovars Manilae, Losbanos, Pyrogenes, and Canicola. Seven to 14 days after infection, urine specimens were collected by aseptic aspiration from the urinary bladders of the dead or sacrificed hamsters. A part of the urine sample was then cultured in modified Korthof's medium and observed until 1 month of incubation at 30°C. Urine was also used to find the optimum conditions for sample treatment. Forty-four urine samples from patients with suspected leptospirosis and 14 samples from healthy persons were obtained from the College of Public Health, University of Philippines—Manila and Kyushu University, respectively. These urine samples were tested by dipstick assay, immunochromatographic assay, and PCR.

Pretreatment of urine. Optimization of urine treatment was performed using *Leptospira*-infected- and noninfected-hamster urine samples treated using several methods: (i) boiling for 5 min (20), (ii) centrifugation at 20,000 × *g* for 15 min (21) followed by resuspension of precipitate with phosphate buffer (pH 7.2), (iii) ultrafiltration and concentration (22), and (iv) boiling for 5 min, centrifugation at 1,000 × *g* for 15 min, and centrifugation (for ultrafiltration and concentration) of supernatant. Ultrafiltration and concentration of urine in methods (iii) and (iv) were performed two times by filtering the supernatant with an Amicon Ultra 30K (Millipore) filter and collecting the filtrate. It was then filtered and concentrated using an Amicon Ultra 10K device (Millipore). The concentrate was resuspended using 10 mM phosphate buffer (pH 7.2). Centrifugation speed (14,000 × *g*) and time (i.e., 10 min and 15 min for Amicon 30K and 10K, respectively) were selected according to the manufacturer's instructions. As a result, urine samples were concentrated 10 times. Those samples were then tested using the dipstick assay to determine the best conditions for urine treatment for analyzing urine samples from patients and hamsters.

Microscopic agglutination test. MAT of serum samples from the same patients with urine samples was performed using the standard method (16, 23). The endpoint titer was the serum dilution that gave ≥50% agglutination at a titer of ≥1:400.

Gold conjugation of MAbs. Gold colloid with a 40-nm diameter (BB International, United Kingdom) was adjusted to pH 9 using 0.1 M K_2CO_3 (24) and then was mixed with 23 μg/ml purified MAb. After 1 h of incubation at room temperature with slow mixing, 0.1% of skim milk was added to block unconjugated sites and was incubated for 10 min. Gold-conjugated antibodies were separated by centrifugation at 6,000 × *g* for 1 hour, washed two times with 2 mM borate buffer (pH 7.2), and kept in 10% initial volume of storage buffer (2 mM borate buffer [pH 7.2], 0.1% skim milk) (25).

Preparation of dipstick and immunochromatography-based lateral flow assays (LFA). (i) Membrane. Nitrocellulose membrane HF240 (Millipore) was cut into 0.5-cm widths. Two micrograms of 1H6 MAb in 2 μl was dropped on the test (T) area, while 2 μg of goat anti-mouse IgG antibody (Rockland) in 2 μl was dropped on the internal control (IC) area. The membrane was dried in a desiccator for 1 to 2 h at 37°C. In order to block the unconjugated areas, the membrane was dipped in 10 mM phosphate buffer (pH 7.2) containing 1% skim milk for 15 min and was washed two times in the same buffer to wash off any excessive blocking reagent. The membrane was then dried overnight at room temperature.

(ii) Conjugate pad. Glass fiber conjugate pads (Millipore), 1 by 0.5 cm, were dipped in gold-conjugated antibodies dissolved in 2 mM borate buffer (pH 7.2) plus 5% sucrose. The pad was then dried at 37°C for 2 h (26).

(iii) Sample pad. Sample pads were treated according to Shim et al. (27), with some modifications. Cellulose fiber sample pads (Millipore), 1.5 by 0.5 cm, were dipped into the sample pad buffer (50 mM borate buffer [pH 7.2], 5% sucrose, 0.5% Tween 20, 5% dextran, and 0.1% skim milk) and then dried at 50°C.

(iv) Dipstick assay. Forty microliters of pretreated urine sample was put into a 96-well microtiter plate. Twelve microliters of gold-conjugated antibodies was mixed with the sample. The mixture was incubated for 15 min at room temperature. The dipsticks were dipped into the mixture, and the results were observed for a maximum of 15 min. Positive test results were indicated by two red spots (in the internal control [IC] area and test area), while negative test results were shown by only one red spot (in the internal control area). The test was invalid if no red spot appeared in the IC area.

(v) ICG-based LFA. One hundred microliters of sample was dropped onto the sample pad. The results were observed for a maximum of 15 min. The interpretation of results was the same as in the dipstick assay.

Sensitivity and specificity of the tests. *Leptospira* strains were cultivated in modified Korthof's medium for several days and were counted using a Thoma counting chamber. The leptospiral culture was centrifuged at 10,000 × *g* for 20 min. Cultures of *Streptococcus* spp., *Enterococcus* spp., *Legionella* spp., *E. coli*, and *Pseudomonas* spp. were centrifuged at 9,000 × *g* for 20 min. The pellets were washed and resuspended in 10 mM phosphate buffer (pH 7.2). The assay detection limit was tested using dilutions of 10^7 to 10^1 cells of *L. interrogans* serovar Manilae strain K64, a local isolate from the Philippines (23). The cells were sonicated in the same buffer and were used for the sensitivity test. The specificity of the assays was tested using the bacterial species listed in Table 1.

PCR. Urine samples were centrifuged at 13,000 × *g* for 10 min. Genomic DNA was purified from the pellet using the Illustra bacterial genomic prep mini spin kit (GE Healthcare, United Kingdom) according to the manufacturer's instructions. Two kinds of PCR assays were performed that targeted the *flaB* gene (793 bp), which is specific for pathogenic *Leptospira* spp. (28), and the *rrl* gene (482 bp), which is specific for *Leptospira* genus (29). The primers used in this experiment were specific for the *flaB* gene (L-flaB-F1 [5′-TCTCACCGTTCTCTAAAGTTCAAC-3′] and L-flaB-R1 [5′-CTGAATTCGGTTTCATATTTGCC-3′]) (27) and for the *rrl* gene (rrlF [5′-GACCCGAAGCCTGTCGAG-3′] and rrlR [5′-GCCATGCTTAGTCCCGATTAC-3′]) (29) of *Leptospira* spp. *flaB* was amplified under the following conditions: 40 cycles of denaturing at 94°C for 20 s, annealing at 50°C for 30 s, extension at 72°C for 60 s, and final extension at 72°C for 5 min. The PCR condition for *rrl* was determined according to Léon et al. (29). PCR products were electrophoresed using 0.7% agarose gel and visualized using ethidium bromide stain.

Statistical analysis. The minimum sample size required for the present study was estimated allowing for an error of 10% and for assumed sensitivities and specificities of 85% each. To test the differences between ICG-based LFA and dipstick assay, when compared with the gold standard, data were analyzed using the Wilcoxon test using SPSS 17.0.

Human and animal ethics. The Ethics Committee on Animal Experiment at the Faculty of Medical Sciences, Kyushu University, reviewed and approved all the animal experiments in this study. These experiments were done based on the conditions stated in the Guideline for Animal Experiments of Kyushu University (law 8 no. 105) and notification no. 6 of the Government of Japan. Human samples were obtained after verbal and written explanations of the study and procedures and after the consent of the subjects or their guardian(s) (for patients with suspected leptospirosis) was obtained. The Ethics Committee of Kyushu University and University of the Philippines—Manila approved the conduct of this study on samples from humans.

RESULTS

Characterization of monoclonal antibody. SDS-PAGE analysis of purified monoclonal antibodies was carried out. Under nonreducing conditions (i.e., without the addition of dithiothreitol [DTT]), a band of antibody was seen around 170 kDa, while under reducing conditions, a heavy chain was noted around 60 kDa and a light chain was seen at 25 kDa (30). Based on these results, the antileptospiral LPS antibody was successfully purified by the method used here. LPS was extracted, purified, and electrophoresed as shown in Fig. 1 (upper panel). Immunoblotting with 1H6 MAb to the purified LPS showed that the antibody was specific to the LPS of *L. interrogans* (Fig. 1, lower panel). We determined that the purified MAb was of class IgG3 based on typing result.

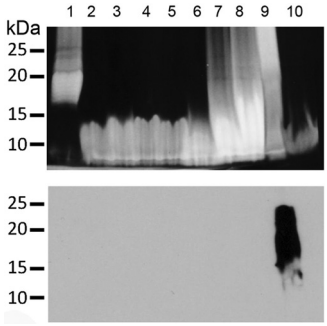

FIG 1 Detection of MAb (1H6)-reactive antigen in *Leptospira* and other bacteria. SDS-PAGE of bacterial LPS with Pro-Q Emerald staining (upper panel) and immunoblotting with MAb-1H6 (lower panel). Lane 1, *Legionella pneumophila*; lanes 2 and 3, uropathogenic *E. coli* C16 and C17; lanes 4 and 5, *Serratia marcescens* J1 and J5; lane 6, *Streptococcus pyogenes*; lane 7, *Borrelia burgdorferi* B31; lane 8, *Borrelia afzelii* P/Gau; lane 9, *L. interrogans* serovar Hebdomadis; and lane 10, bovine serum albumin (BSA).

Optimization of conjugation of gold with monoclonal antibody. Preliminary experiments were performed to find the optimal combination of antibodies (Abs) using both polyclonal and monoclonal antibodies. When we used polyclonal antibodies purified from guinea pigs infected with five strains of *Leptospira*, we found many false-positive results. Therefore, we tried to use one monoclonal antibody as a capture Ab and immobilized Ab, which showed better results than other combinations of antibodies (data not shown).

Preliminary experiments were also carried out to determine the optimum conditions for conjugating antibodies with gold colloid. Prior to the conjugation of gold colloid and monoclonal antibody, titration was performed to determine the least amount of antibody that could stabilize gold colloid. Using the method described in the study by Beesley (24), with some modifications, the minimal concentration of antibody was determined (data not shown) and 23 μg/ml (four times the minimum concentration) of antibody was used for conjugation with gold colloid. After preliminary experiments using different concentrations, the dose of immobilized antibody was determined to be 2 μg (data not shown).

Sensitivity and specificity of the dipstick assay. A positive result in the dipstick assay was seen with 10^7 to 10^6 cells. Meanwhile, with 10^5 cells, the antigen could no longer be detected. Therefore, the detection limit of the dipstick assay was determined to be 10^6 cells/ml when disrupted cells of *Leptospira* were used.

The specificity of the dipstick assay was tested against several bacterial species, which are listed in Table 1. Results showed that the assay has a high specificity for all strains of *Leptospira* used in this study, whether pathogenic or not (*L. interrogans* serovars Autumnalis, Canicola, Manilae, Grippotyphosa, and Icterohaemorrhagiae *Leptospira borgpetersenii* serovars Poi, Tarassovi, and Javanica, and *Leptospira biflexa* serovar Patoc). However, the assay was negative when other bacterial species were used, such as those whose antigens are known to be excreted in urine (*L. pneumophila* and *S. pneumoniae*), and in uropathogenic bacteria (*E. coli*, *Enterococcus faecalis*, and *P. aeruginosa*). The results showed that this assay could discriminate *Leptospira* from other bacteria, but it

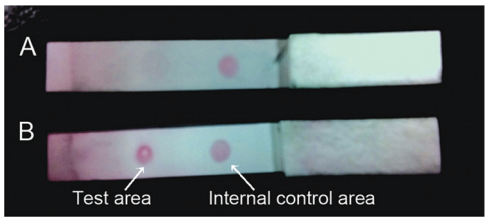

FIG 2 Dipstick assay using hamster urine. (A) Negative result; (B) positive result.

TABLE 2 Comparison of dipstick assay results with culture method using infected-hamster urine[a]

	Culture method		
Dipstick	Positive	Negative	Total
Positive	22	6	28
Negative	7	11	18
Total	29	17	46

[a] Sensitivity, 0.76 (76%) (95% CI, 0.63–0.89); specificity, 0.65 (65%) (95% CI, 0.51–0.79). Values are number of samples.

could not discriminate pathogenic from nonpathogenic *Leptospira*.

Determination of the best conditions for sample treatment. Treatment of urine samples was carried out to determine the conditions that might increase the sensitivity and specificity of the assays. Treatment was necessary because of nonspecific bindings based on the immunoblotting results of urine samples from patients with suspected leptospirosis (data not shown).

The results of the dipstick assay showed that leptospiral antigen could be detected in the urine of *Leptospira*-infected hamsters after using all sample treatments. However, when we used uninfected-hamster urine samples that were not treated, were only boiled, or were only centrifuged, the dipstick assay was positive. In a study by Doskeland and Berdal (31), boiling urine eliminated nonspecific reactions by substances in it. In our study, however, boiling alone was not enough to eliminate these reactions, probably due to a heat-stable substance that bound to gold-conjugated antibody. Centrifugation at 20,000 × *g* was used to increase the sensitivity of the assay, but it did not eliminate the nonspecific substances that interfered with the result. A combination of boiling and centrifugal filtration, however, eliminated the nonspecific reactions in uninfected-hamster urine samples. Based on the results of our study and those of Cinco et al. (32), the lipopolysaccharide of *Leptospira* has a size between 10 and 30 kDa; therefore, we used two kinds of ultrafilters to obtain the antigen of this size. Using this method, we successfully eliminated the nonspecific substance in uninfected-hamster urine. These methods, therefore, might be used to pretreat the urine samples of infected hamsters and patients with suspected leptospirosis prior to analysis using the two diagnostic methods.

Dipstick assay for *Leptospira*-infected hamster urine. The minimum sample size calculated for this study was 13. Forty-six urine samples of *Leptospira*-infected hamsters were collected and stored at −20°C prior to testing. The optimum conditions of urine treatment mentioned above were used prior to analyzing the infected-hamster urine using the dipstick assay. Figure 2 shows the representative results of the dipstick assay.

Results of the dipstick assay showed that 28 of 46 samples of hamster urine (60.9%) were positive, while 29 of 46 samples (63.1%) were positive in culture. The sensitivity and specificity of the dipstick assay were calculated by comparing the results with the gold standard (i.e., culture) (Table 2) and were found to be 76% and 65%, respectively. Some discrepancies between the dipstick assay and culture results were observed.

Dipstick and ICG-based LFA for urine of humans with suspected leptospirosis. Urine samples from patients with suspected leptospirosis were collected, stored at −20°C prior to testing, and then tested using the dipstick assay, ICG-based LFA, and PCR. Serum samples of patients were tested using MAT. For the human urine samples, we performed the dipstick assay, ICG-based LFA, and PCR, because the amounts of the samples were enough to perform all 3 methods. The representative results of ICG-based LFA are shown in Fig. 3. For determining the specificity of the assays, we also tested urine samples from healthy persons using the dipstick assay and ICG-based LFA. PCR was used to confirm the results of the 2 assays.

flaB PCR was more sensitive in detecting *Leptospira* in urine than was *rrl*. We found discrepancies between the results of MAT and PCR. Since these 2 methods were used as gold standards, to calculate the sensitivity and specificity of the dipstick assay and ICG-based LFA, the gold standard was considered positive when either of these two methods was positive.

The assays developed in this study could detect the antigen from the first day after the onset of illness, as shown in Table 3. Compared to the gold standard (PCR and/or MAT), different results of both assays were found mostly by the fifth day after onset in 19 samples and became relatively consistent from the sixth day on. By the fifth day after onset, we found 6 false-negative and 3 false-positive results for the dipstick assay and 3 false-negative and 2 false-positive results for the ICG-based LFA. After the sixth day, we found only 1 false-positive result for ICG-based LFA and 1 false-negative result for both assays with an unknown time of disease onset. From 44 samples from patients with suspected leptospirosis, 4 samples were negative for all assays; therefore, we deduced that these patients were not infected by *Leptospira*. The results from the urine samples of patients with suspected leptospirosis and healthy persons showed that 35 of 58 samples (60.3%) were positive for MAT or PCR. Thirty-four of the 58 samples (58.7%) were positive in the dipstick assay and ICG-based LFA, as shown in Tables 4 and 5. The sensitivity and specificity of the dipstick assay were 80% and 74%, respectively. The results of the

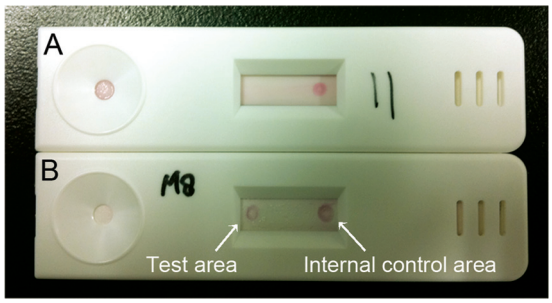

FIG 3 ICG-based LFA using human urine. (A) Negative result; (B) positive result.

TABLE 3 Results of MAT, PCR, dipstick, and ICG-based LFA on urine samples from suspected-leptospirosis patients

Patient no.	Day of urine collection after onset of illness	Assay result				MAT serovars (titers)
		Dipstick	ICG-based LFA	PCR	MAT	
1	1	+	+	−	+	Copenhageni (1:400)
2	2	+	−	+	−	
3	2	+	+	+	+	Patoc (1:3,200)
4	2	+	+	+	+	Poi (1:400)
5	3	−	−	−	−	
6	3	+	−	−	−	
7	3	−	−	+	−	
8	3	+	+	−	−	
9	3	+	+	+	+	Patoc (1:1,600), Poi (1:400)
10	3	+	+	+	+	Patoc (1:400)
11	4	−	−	−	+	Manilae (1:800), Patoc (1:800)
12	4	−	+	−	+	Poi (1:400)
13	4	−	+	−	+	Copenhageni (1:800), Patoc (1:400)
14	4	+	+	+	+	Patoc (1:1,600)
15	4	+	+	−	+	Patoc (1:400)
16	4	+	+	−	−	
17	5	−	+	+	−	
18	5	+	+	+	+	Pyrogenes (1:400)
19	5	−	+	+	−	
20	6	+	+	+	+	Patoc (1:400)
21	6	+	+	+	+	Canicola (1:800)
22	6	+	+	+	−	
23	7	−	−	−	−	
24	7	+	+	−	+	Patoc (1:6,400)
25	8	−	−	−	−	
26	8	+	+	+	+	Patoc (1:6,400)
27	8	+	+	−	+	Patoc (1:800)
28	8	+	+	+	+	Patoc (1:1,600), Copenhageni (1:800), Semaranga (1:800)
29	9	+	+	−	+	Poi (1:800), Patoc (1:400)
30	10	−	+	−	−	
31	12	+	+	−	+	Losbanos (1:400)
32	13	+	+	+	+	Copenhageni (1:400), Losbanos (1:400)
33	14	−	−	−	−	
34	15	+	+	+	+	Patoc (1:3,200)
35	18	+	+	−	+	Ratnapura (1:1,600), Poi (1:800), Patoc (1:800), Semaranga (1:400)
36	21	−	−	−	−	
37	22	+	+	+	+	Patoc (1:3,200), Pyrogenes (1:800)
38	52	+	+	−	+	Ratnapura (1:1,600), Copenhageni (1:400), Patoc (1:400), Semaranga (1:400)
39	68	+	+	+	+	Patoc (1:6,400), Canicola (1:800), Ratnapura (1:800), Semaranga (1:800)
40	Unknown	+	+	+	+	Patoc (1:800)
41	Unknown	+	+	−	+	Patoc (1:800), Losbanos (1:400)
42	Unknown	+	+	+	+	Semaranga (1:1,600), Patoc (1:400)
43	Unknown	−	−	+	−	
44	Unknown	+	+	+	−	

TABLE 4 Comparison of dipstick assay results with PCR (human urine) and/or MAT (human serum)[a]

Dipstick	PCR and/or MAT		
	Positive	Negative	Total
Positive	28	6	34
Negative	7	17	24
Total	35	23	58

[a] Sensitivity, 0.8 (80%) (95% CI, 0.7–0.9); specificity, 0.74 (74%) (95% CI, 0.63–0.85). Values are number of samples.

ICG-based LFA and gold standard are shown in Table 5, and the sensitivity and specificity were 89% and 87%, respectively. The sensitivity and specificity of the ICG-based LFA were higher than those of the dipstick assay. The Wilcoxon analysis also showed that the ICG-based LFA was not significantly different from the gold standard ($P = 0.3557$, $\alpha = 0.05$). The efficiencies of the dipstick assay and ICG-based LFA were 78% and 88%, respectively. These results showed that the ICG-based LFA is better than the dipstick assay due to its higher sensitivity, specificity, and efficiency.

TABLE 5 Comparison of ICG-based LFA results with PCR (human urine) and/or MAT (human serum)[a]

ICG-based LFA	PCR and/or MAT		
	Positive	Negative	Total
Positive	31	3	34
Negative	4	20	24
Total	35	23	58

[a] Sensitivity, 0.89 (89%) (95% CI, 0.78–0.96); specificity, 0.87 (87%) (95% CI, 0.8–0.96). Values are number of samples.

DISCUSSION

Leptospirosis is an infectious disease prevailing around the world. The diagnosis of leptospirosis is mainly determined by MAT, culture, and PCR methods (16). However, the limitations of these assays (i.e., they are laborious, time-consuming, and expensive) brought out the need for another simple, fast, and inexpensive diagnostic method. In this study, we tried to develop a rapid diagnostic assay (i.e., dipstick and ICG-based LFA) for leptospirosis which might fulfill these requirements and be applicable in all countries. Nowadays, the ICG assay for leptospirosis, which is used as one of the diagnostic methods, is mainly used for the detection of antibodies (9, 33, 34). However, these assays are difficult to use for early diagnosis because of their low sensitivities and the fact that the immune response might not have developed enough to be detected during the early stages of infection. The antigen of *Leptospira* might be a good target for detection, particularly during the early phase of infection, because it is excreted in the urine from day 6 postinfection onward (10, 35). Therefore, we tried to develop ICG-based methods that aimed to detect the antigen of *Leptospira* using anti-*Leptospira* antibodies.

The monoclonal antibody 1H6, which was used in this study, has been characterized as against the lipopolysaccharide of *Leptospira*. This antibody was tested for purified LPS of several bacterial species prior to the development of the diagnostic kit. The monoclonal antibody was reactive to the 12-kDa LPS of *Leptospira*, as seen in Fig. 1. Leptospiral LPS is known to have high antigenicity, and therefore, anti-LPS antibodies are found in human and animal sera (36).

Preliminary experiments using combinations of polyclonal and monoclonal antibodies showed false-positive results due to possible nonspecific reactions. Polyclonal antibodies produced by the immunization of whole bacteria resulted in low specificity (37). By using only one kind of monoclonal antibody, we achieved better results than with a combination of polyclonal and monoclonal antibodies. We hypothesized that there are multiple epitopes in one molecule of LPS and that the capture antibody bound to one of the epitopes while the immobilized antibody bound to unoccupied epitopes. Usage of a single antibody for capture and immobilized antigens was also reported in ICG-based methods, which were successful in detecting *Campylobacter* antigens (38) and botulinum neurotoxin (39). This single antibody was adapted for our dipstick assay and ICG-based LFA, which were tested for sensitivity and specificity against *Leptospira* spp. and other bacterial species. For sensitivity, we have tested different concentrations of *Leptospira* culture, and the detection limit was 10^6 cells/ml. This result was almost the same as the detection limit of that achieved with the immunochromatographic assay for other bacterial species (25, 37). For a specificity assay, we tested this single antibody system against several strains of *Leptospira* and bacteria that are commonly found in urine or are known to be the causative agents of urinary infection. Results showed that all *Leptospira* spp. could be detected, but the non-*Leptospira* bacteria used in this study could not, which means this assay can discriminate between *Leptospira* antigen and other bacterial antigens.

The method was applied to infected- and noninfected-hamster urine samples and was compared with cultures. Direct applications of the hamster urine always gave false-positive results for noninfected-hamster urine. This might be caused by nonspecific reactions with unknown substances in the urine. Therefore, pretreatment of the urine was necessary to eliminate these substances. Boiling urine for 3 min might cause the liberation of LPS antigen from the naturally formed antigen-antibody complex and increase a specific antigen-antibody reaction (31). However, in this study, pretreatment of urine by boiling only was not enough to eliminate nonspecific reactions. A combination of boiling and concentrating (through ultrafiltration) urine might eliminate nonspecific reactions in uninfected-hamster urine in our study. Concentration and filtration of urine were believed to increase the sensitivity and specificity of diagnostic assays (20). This result was consistent when using human urine (unpublished data). However, further study is necessary in order to create simpler and more-cost-effective methods of pretreating urine samples.

A dipstick assay was applied to the urine samples of 46 infected hamsters. The culture method was used as a comparison method and a gold standard for the calculation of sensitivity and specificity. The culture method is known to be one of the reference diagnostic tests for leptospirosis (4). The sensitivity and specificity of the dipstick assay were 76% and 65%, respectively. These results were quite low when comparing the dipstick assay with the detection of *Leptospira* antibody in serum (40, 41), which showed >90% sensitivity and specificity. This might be caused by the relatively small amount of *Leptospira* antigens in the sample. We have estimated that the detection limit for the dipstick assay is 10^6 cells of *Leptospira*. The concentration of *Leptospira* that is usually found in urine from dogs ranges from 10^1 to 10^6 cells/ml (42). Monahan et al. (10) reported that rats could excrete high concentrations of *Leptospira* (10^7 cells/ml) after 3 weeks of infection. Because the concentration of *Leptospira* varies among animals, this kit might not be applicable for samples with leptospiral concentrations below the detection limit. For infected-hamster urine, we tested using the dipstick assay only, due to the limited volume of samples.

Urine samples from 44 patients with suspected leptospirosis and 14 healthy humans were tested using the dipstick assay and ICG-based LFA. PCR and MAT were also performed and were used as gold standards because we found several discrepancies between PCR and MAT results, as shown in Table 3. Some samples were positive by PCR but negative by MAT. This might be because some of the serum samples were obtained during the acute phase of illness; therefore, the immune response elicited was not enough to be detected using MAT. We also found that some PCR-negative samples were diagnosed as positive by MAT. Although we do not have evidence for this, we think it might be caused by *Leptospira*, which is not always excreted in the urine of leptospirosis patients, or that the number of *Leptospira* in the urine was below the detection limit of PCR. Another possible cause of this discrepancy is that antibiotics used by the patients eradicated *Leptospira* in the

kidneys, making it undetectable by PCR. Recently, it has been hypothesized that MAT is an imperfect gold standard for leptospirosis (43). Therefore, a combination of PCR and MAT as the gold standard might more precisely predict the true sensitivity and specificity of the diagnostic assays that we developed.

The results in Table 3 show that both of our assays could detect the leptospiral antigen in urine from the first day after the onset of illness. However, until the fifth day after onset, some results of the dipstick assay and ICG-based LFA were different from those with the gold standard. This might be because during that time, the antigen or intact bacteria that are excreted in urine are still low in number or are excreted intermittently. Saengjaruk et al. (9) also showed varied results of antigen detection in urine samples collected consecutively, which is caused by the intermittent shedding of *Leptospira* in urine. Results for urine samples from infected patients, tested after the sixth day postonset, showed relatively consistent results with those of the gold standard, which might be caused by an increasing number and continuous shedding of *Leptospira* or its antigen. Prolonged shedding of leptospires in the urine (collected at the 52nd and 68th days after onset of illness) was also found in two patients who tested positive in the two assays. For the urine samples from healthy persons, the dipstick assay showed two false positives, while ICG-based LFA showed results consistent with those of the gold standard.

The sensitivity and specificity of the dipstick assay used for human urine samples were 80% and 74%, respectively, higher than those (76% and 65%, respectively) for the hamster urine samples. Although we do not have evidence yet, we think that this is because the concentration of *Leptospira* antigen in hamster urine was mostly below the detection limit and was lower than that in human urine. However, the sensitivity and specificity of the dipstick assay for human urine were lower than those of the ICG-based LFA, which were 89% and 87%, respectively. This might be caused by the treatments of the sample pad and the conjugate pad. The presence of dextran and the nonionic detergent Tween 20 at low concentrations in the sample pad might enhance resolubilization of the conjugate, reduce nonspecific reactions, and minimize adsorption of the analyte on membrane (26). The addition of sucrose, which is known as a preservative and resolubilization agent (26), to the conjugate pad made the gold conjugate more stable and flow better. Even though the overall sensitivity and specificity of the ICG-based LFA are still <90%, they are still higher than those of the IgM dipstick assay (9), which is often used in an initial screening for leptospirosis. In summary, we have developed assays that can detect the presence of *Leptospira* antigen in the urine of humans and animals and can discriminate it from other bacterial antigens; however, this assay is not able to identify the infecting serovar of *Leptospira*. We have also developed new approaches for eliminating nonspecific reactions and concentrating urine. This is the first study that could detect *Leptospira* antigen in human and hamster urine by the use of immunochromatography-based assays with good sensitivity and specificity. Further development is needed in the pretreatment of samples and applications for mass production in order to be applicable to resource-poor areas, where leptospirosis is usually endemic.

ACKNOWLEDGMENTS

This study was supported by a grant in a scheme of the Science and Technology Research Partnership for Sustainable Development (SATREPS) Program from the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA).

We thank all the persons who helped in sample collection (staff and students of the Department of Bacteriology, Graduate School of Medical Sciences, Kyushu University, and the staff of the project for the Prevention and Control of Leptospirosis in the Philippines, Department of Medical Microbiology, College of Public Health, University of the Philippines, Manila, Philippines); Suminori Kono (Department of Preventive Medicine, Kyushu University); and Fase Badriah Zain for advice on statistical analysis. Dian Widiyanti would also like to extend appreciation to the Ministry of Education, Culture, Sport, Science, and Technology of Japan (MEXT) for her scholarship.

REFERENCES

1. **Faine S, Adler B, Bolin C, Perolat P.** 2000. *Leptospira* and leptospirosis, 2nd ed. Medisci Press, Melbourne, Australia.
2. **Johnson RC.** 1996. *Leptospira. In* Baron S (ed), Medical microbiology, 4th ed. University of Texas Medical Branch at Galveston, Galveston, TX. http://www.ncbi.nlm.nih.gov/books/NBK8451.
3. **Ko AI, Goarant C, Picardeau M.** 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. **7**:736–747.
4. **World Health Organization.** 2010. Report of the first meeting of the Leptospirosis Burden Epidemiology Reference Group. WHO Press, Geneva, Switzerland.
5. **Senthilkumar T, Subathra M, Phil M, Ramadass P, Ramaswamy V.** 2008. Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay. Indian J. Med. Microbiol. **26**:45–49.
6. **Gussenhoven GC, van der Hoorn MA, Goris MG, Terpstra WJ, Hartskeerl RA, Mol BW, van Ingen CW, Smits HL.** 1997. LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. J. Clin. Microbiol. **35**:92–97.
7. **Wagenaar J, Zuerner RL, Alt D, Bolin CA.** 2000. Comparison of polymerase chain reaction assays with bacteriologic culture, immunofluorescence, and nucleic acid hybridization for detection of *Leptospira borgpetersenii* serovar Hardjo in urine of cattle. Am. J. Vet. Res. **61**:316–320.
8. **Smits HL, van der Hoorn MA, Goris MG, Gussenhoven GC, Yersin C, Sasaki DM, Terpstra WJ, Hartskeerl RA.** 2000. Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. J. Clin. Microbiol. **38**:1272–1275.
9. **Saengjaruk P, Chaicumpa W, Watt G, Bunyaraksyotin G, Wuthiekanun V, Tapchaisri P, Sittinont C, Panaphut T, Tomanakan K, Sakolvaree Y, Chongsa-Nguan M, Mahakunkijcharoen Y, Kalambaheti T, Naigowit P, Wambangco MA, Kurazono H, Hayashi H.** 2002. Diagnosis of human leptospirosis by monoclonal antibody-based antigen detection in urine. J. Clin. Microbiol. **40**:480–489.
10. **Monahan AM, Callanan JJ, Nally JE.** 2008. Proteomic analysis of *Leptospira interrogans* shed in urine of chronically infected hosts. Infect. Immun. **76**:4952–4958.
11. **Koizumi N, Nakajima C, Harunari T, Tanikawa T, Tokiwa T, Uchimura E, Furuya T, Mingala CN, Villanueva MA, Ohnishi M, Suzuki Y.** 2012. A new loop-mediated isothermal amplification method for rapid, simple, and sensitive detection of *Leptospira* spp. in urine. J. Clin. Microbiol. **50**:2072–2074.
12. **Murray CK, Gray MR, Mende K, Parker TM, Samir A, Rahman BA, Habashy EE, Hospenthal DR, Pimentel G.** 2011. Use of patient-specific *Leptospira* isolates in the diagnosis of leptospirosis employing microscopic agglutination testing (MAT). Trans. R. Soc. Trop. Hyg. **105**:209–213.
13. **Domínguez J, Galí N, Matas L, Pedroso P, Hernández A, Padilla E, Ausina V.** 1999. Evaluation of a rapid immunochromatographic assay for the detection of *Legionella* antigen in urine samples. Eur. J. Clin. Microbiol. Infect. Dis. **18**:896–898.
14. **Rosón B, Fernández-Sabé N, Carratalà J, Verdaguer R, Dorca J, Manresa F, Gudiol F.** 2004. Contribution of a urinary antigen assay (Binax NOW) to the early diagnosis of pneumococcal pneumonia. Clin. Infect. Dis. **38**:222–226.
15. **Chanteau S, Dartevelle S, Mahamane AE, Djibo S, Boisier P, Nato F.** 2006. New rapid diagnostic tests for *Neisseria meningitidis* serogroups A, W135, C, and Y. PLoS Med. **3**:e337. doi:10.1371/journal.pmed.0030337.

16. **World Health Organization.** 2003. Human leptospirosis: guidance for diagnosis, surveillance and control. World Health Organization and International Leptospirosis Society, Geneva, Switzerland.
17. **Masuzawa T, Kumagai M, Shimizu T, Yanagihara Y.** 1988. Classification of *Leptospira interrogans* serovar Lai strain 017 by using monoclonal antibodies. J. Clin. Microbiol. **26**:2332–2337.
18. **Harlow E, Lane DP.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. **Bligh EG, Dyer WJ.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
20. **Domínguez JA, Manterola JM, Blavia R, Sopena N, Belda FJ, Padilla E, Giménez M, Sabrià M, Morera J, Ausina V.** 1996. Detection of *Legionella pneumophila* serogroup 1 antigen in nonconcentrated urine and urine concentrated by selective ultrafiltration. J. Clin. Microbiol. **34**:2334–2336.
21. **Chirathaworn C, Chantaramalai T, Sereemaspun A, Kongthong N, Suwancharoen D.** 2009. Detection of *Leptospira* in urine using anti-*Leptospira*-coated gold nanoparticles. Comp. Immunol. Microbiol. Infect. Dis. **34**:31–34.
22. **Blanco S, Prat C, Pallarés MA, Matas L, Domínguez J.** 2004. Centrifugal ultrafiltration method for rapid concentration of *Legionella pneumophila* urinary antigen. J. Clin. Microbiol. **42**:4410.
23. **Villanueva SY, Ezoe H, Baterna RA, Yanagihara Y, Muto M, Koizumi N, Fukui T, Okamoto Y, Masuzawa T, Cavinta LL, Gloriani NG, Yoshida S.** 2010. Serologic and molecular studies of *Leptospira* and leptospirosis among rats in the Philippines. Am. J. Trop. Med. Hyg. **82**:889–898.
24. **Beesley J.** 1989. Colloidal Gold: a new perspective for cytochemical marking. Oxford University Press, Oxford, United Kingdom.
25. **Shim WB, Choi JG, Kim JY, Yang ZY, Lee KH, Kim MG, Ha SD, Kim KS, Kim KY, Kim CH, Ha KS, Eremin SA, Chung DH.** 2007. Production of monoclonal antibody against *Listeria monocytogenes* and its application to immunochromatography strip test. J. Microbiol. Biotechnol. **17**:1152–1161.
26. **EMD Millipore Corporation.** 2008. Rapid lateral flow test strips: consideration for product development. EMD Millipore Corporation, Billerica, MA. http://www.millipore.com/techpublications/tech1/tb500en00.
27. **Shim WB, Yang ZY, Kim JY, Choi JG, Je JH, Kang SJ, Kolosova AY, Eremin SA, Chung DH.** 2006. Immunochromatography using colloidal gold-antibody probe for the detection of atrazine in water samples. J. Agric. Food Chem. **54**:9728–9734.
28. **Kawabata H, Dancel LA, Villanueva SY, Yanagihara Y, Koizumi N, Watanabe H.** 2001. flaB-polymerase chain reaction (flaB-PCR) and its restriction fragment length polymorphism (RFLP) analysis are an efficient tool for detection and identification of *Leptospira* spp. Microbiol. Immunol. **45**:491–496.
29. **Léon A, Pronost S, Tapprest J, Foucher N, Blanchard B, André-Fontaine G, Laugier C, Fortier G, Leclercq R.** 2006. Identification of pathogenic *Leptospira* strains in tissues of a premature foal by use of polymerase chain reaction analysis. J. Vet. Diagn. Invest. **18**:218–221.
30. **Colman R, Hussain A, Goodall M, Young SP, Pankhurst T, Lu X, Jefferis R, Savage COS, Williams JM.** 2007. Chimeric antibodies to proteinase 3 of IgG1 and IgG3 subclasses induce different magnitudes of functional responses in neutrophils. Ann. Rheum. Dis. **66**:676–682.
31. **Doskeland SO, Berdal BP.** 1980. Bacterial antigen detection in body fluids: methods for rapid antigen concentration and reduction of nonspecific reactions. J. Clin. Microbiol. **11**:380–384.
32. **Cinco M, Banfi E, Panfili E.** 1986. Heterogeneity of lipopolysaccharide banding patterns in *Leptospira* spp. J. Gen. Microbiol. **132**:1135–1138.
33. **Sehgal SC, Vijayachari P, Sugunan AP, Umapathi T.** 2003. Field application of Lepto lateral flow for rapid diagnosis of leptospirosis. J. Med. Microbiol. **52**:897–901.
34. **Smits HL, Eapen CK, Sugathan S, Kuriakose M, Gasem MH, Yersin C, Sasaki D, Pujianto B, Vestering M, Abdoel TH, Gussenhoven GC.** 2001. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin. Diagn. Lab. Immunol. **8**:166–169.
35. **Nally JE, Monahan AM, Miller IS, Bonilla-Santiago R, Souda P, Whitelegge JP.** 2011. Comparative proteomic analysis of differentially expressed proteins in the urine of reservoir hosts of leptospirosis. PLoS One **6**:e26046. doi:10.1371/journal.pone.0026046.
36. **Adler B, de la Peña Moctezuma A.** 2010. *Leptospira* and leptospirosis. Vet. Microbiol. **140**:287–296.
37. **Ju Y, Hao HJ, Xiong GH, Geng HR, Zheng YL, Wang J, Cao Y, Yang YH, Cai XH, Jiang YQ.** 2010. Development of colloidal gold-based immunochromatographic assay for rapid detection of *Streptococcus suis* serotype 2. Vet. Immunol. Immunopathol. **133**:207–211.
38. **Kawatsu K, Kumeda Y, Taguchi M, Yamazaki-Matsune W, Kanki M, Inoue K.** 2008. Development and evaluation of immunochromatographic assay for simple and rapid detection of *Campylobacter jejuni* and *Campylobacter coli* in human stool specimens. J. Clin. Microbiol. **46**:1226–1231.
39. **Chiao DJ, Shyu RH, Hu CS, Chiang HY, Tang SS.** 2004. Colloidal gold-based immunochromatographic assay for detection of botulinum neurotoxin type B. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **809**:37–41.
40. **Dey S, Mohan CM, Ramadass P, Nachimuthu K.** 2007. Recombinant antigen-based dipstick ELISA for the diagnosis of leptospirosis in dogs. Vet. Rec. **160**:186–188.
41. **Hatta M, Smits HL, Gussenhoven GC, Gooskens J.** 2000. Introduction of a rapid dipstick assay for the detection of *Leptospira*-specific immunoglobulin m antibodies in the laboratory diagnosis of leptospirosis in a hospital in Makassar, Indonesia. Southeast Asian J. Trop. Med. Public Health **31**:515–520.
42. **Rojas P, Monahan AM, Schuller S, Miller IS, Markey BK, Nally JE.** 2010. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. Eur. J. Clin. Microbiol. Infect. Dis. **29**:1305–1309.
43. **Limmathurotsakul D, Turner EL, Wuthiekanun V, Thaipadungpanit J, Suputtamongkol Y, Chierakul W, Smythe LD, Day NP, Cooper B, Peacock SJ.** 2012. Fool's gold: why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. Clin. Infect. Dis. **55**:322–331.